NASA TECHNICAL MEMORANDUM

(93)

REPORT NO. 53891

EVALUATION OF ALCOHOL SPORULATION METHOD

By Manufacturing Research and Technology Division
Manufacturing Engineering Laboratory

September 12, 1969

NASA

George C. Marshall Space Flight Center
Marshall Space Flight Center, Alabama

MSFC - Form 3190 (September 1968)

NASA-GEORGE C. MARSHALL SPACE FLIGHT CENTER

INTERNAL NOTE
IN-R-ME-68-5

Changed to TM X-53891, September 12, 1969

EVALUATION OF ALCOHOL SPORULATION METHOD

By

Manufacturing Research and Development Division
Manufacturing Engineering Laboratory

February 26, 1967

MANUFACTURING ENGINEERING LABORATORY
RESEARCH AND DEVELOPMENT OPERATIONS

EVALUATION OF ALCOHOL SPORULATION METHOD

By

Manufacturing Research and Technology Division
Manufacturing Engineering Laboratory

George C. Marshall Space Flight Center
Huntsville, Alabama

ABSTRACT

An alcohol suspension of Bacillus subtilis variety niger spores was employed as an agent for controlled contamination of stainless steel strips. Test results indicate that this new method will provide a more accurate and controlled level of contamination and will result in cost and time reduction.

This report includes the complete procedure to be followed.

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

ACKNOWLEDGEMENT

This report was prepared by Mrs. A. N. James, Jr. of the Hayes International Corporation under Contract No. NAS8-20083 for the Manufacturing Research and Technology Division, Manufacturing Engineering Laboratory.

Special recognition is granted Mrs. E. A. Quinn, microbiologist, Methods Development Group, Hayes International Corporation, for valuable assistance in compiling information contained in this report. All work was performed under the technical direction of Mr. F. J. Beyerle, Chief, Chemical Processing Section, Mechanical and Chemical Development Branch.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

EVALUATION OF ALCOHOL SPORULATION METHOD

INTRODUCTION

As an integral part of the Manufacturing Engineering Laboratory inhouse sterilization program, procedures and techniques are to be revised and rewritten whenever expedient. Present experiments on comparison of sterilization methods call for sterile pieceparts that have been contaminated to known levels. These samples are subjected to sterilization procedures, then assayed to determine the precise kill level. The adaption of the stainless steel strip analysis that has been used, based on NASA Standard Procedures for Microbiological Examination of Spacecraft Hardware, 1 June 1966, does not produce the precisely controlled level of contamination required.

This report covers the efficiency and effectiveness of a new method for controlled contamination using only spores of the aerobic bacteria Bacillus subtilis variety niger in contaminating solution of ethylene alcohol. The data on each phase of the experiments are presented and evaluated and a complete procedure for the use of this method is described.

EXPERIMENTATION

Materials

1. Sterile stainless steel strips
2. Plastic petri dishes
3. Pipettes (1 ml, 5 ml, 10 ml)
4. 10 ml syringe
5. Automatic pipetting assembly
6. 100% ethyl alcohol
7. Tween 80 (polyoxethylene sorbitan monooleate)
8. Peptone
9. Deionized water
10. Screwcap jars
11. Erhlenmeyer flask
12. Screwcap tubes
13. Agar
14. Forceps

Equipment

1. Laminar flow clean bench
2. Refrigerator
3. Quebec counter
4. Autoclave
5. Weighing scale
6. Incubator

Methods

Proof of Theory. The initial phase of the experiment was designed to demonstrate that this method is effective and to determine the necessity for heat shocking spores. The spores used were from a sample of *Bacillus subtilis* variety *niger* received from Dr. Martin Favero, Public Health Service, Phoenix, Arizona. A 95-percent solution of ethanol was used to contain the spores.

Before using this solution, it was ultrasonicated for 12 minutes to break up any clumps. A series dilution was run to obtain a contaminating solution of 2.46×10^3 spores per milliliter.

Each of 80 sterile stainless steel strips was contaminated with 1 ml of 10^3 spores in alcohol. Petri dishes were left open on the clean bench to allow for evaporation of dilutant (10 minutes).

Dried strips were placed in 50 ml of sterile 1-percent peptone water in sterile screwcap jars and ultrasonicated. Five ml from each bottle were plated out with 20 ml Tripticase Soy Agar (TSA) in 100 by 15 mm petri dishes and incubated aerobically.

The samples were heat shocked for 30 minutes at 80°C. Another 5-ml aliquoit was plated out from each jar; 20 ml sterile, molten TSA were added to each plate. The plates were subsequently incubated aerobically.

All plates, labeled as heat shocked and non-heat shocked were incubated over a period of 72 hours, and counted at 24-, 48-, and 72-hours.

The first 34 strips were left overnight in peptone water in the clean bench, then were ultrasonicated and plated out. After 24 hours all plates that were non-heat shocked had too many colonies to count, whereas the plates that were heat shocked had no colonies. Counts were not continued for non-heat shocked plates, but a total of three colonies was recorded for the heat shocked samples after 72 hours.

The procedure for the remaining 44 strips was carried out as rapidly as possible; no strips remained in the peptone recovery media longer than 2 hours. The average number of colonies per plate was 9.54×10^3 for non-heat shocked samples. For the heat shocked plates, the average 1.23×10^3 colonies per plate.

Results for heat shocked plates were much lower than anticipated. However, any environmental shock (i.e., the change from alcohol solution at 4°C to 38°C peptone water) may stimulate germination of spores if they are in nutrient material. Germination can take place in a minimum of 30 seconds [1].

Comparison of Recovery Media. To compare the efficiency of various types of recovery media, several different solutions were prepared:

1. 1% Tween 80 in deionized water (38°C)
2. 0.1% Tween 80 in deionized water (38°C)
3. 1% peptone in deionized water (38°C)
4. 1% peptone in deionized water (4°C)

The peptone water was used at two different temperatures in order to determine if the environmental shock of transfer from ethyl alcohol suspension at 4°C to the room temperature peptone medium (containing proteins) results in spore germination before heat shock, and if maintaining the peptone water at 4°C prevents this result.

Twenty jars of each type of sterilized recovery solution were prepared, each jar containing 50 ml of solution. Ten of the jars of peptone solution were kept at room temperature while the remaining ten were maintained at 4°C.

Sixty stainless steel strips were inoculated with 2.46×10^3 spores per ml contaminating solution and allowed to dry.

The strips were then placed in the jars of recovery media and allowed to sit at the specified temperature for 2 hours. The samples were then ultrasonicated. Two 5-ml aliquoits from each jar were plated in 20 ml sterile TSA; they were then placed in incubation for 72 hours.

This procedure was carried out to discover the type of recovery solution that gives highest recovery of viable bacteria as well as to determine the amount of germination that occurs before heat shock. Results illustrated in Table I, showed that 1-percent Tween 80 solution had the highest level of recovery; it also had the least germination of spores (as indicated by 50 percent survival). The solution of 0.1-percent Tween 80 showed very low recovery as recorded in Table II. The samples recovered from the peptone at 4°C showed a lower percentage of bacterial growth after heat shock than did the samples from peptone at room temperature (Tables III, IV). This result is not consistent with other findings; it may be that an insufficient number of samples were used to produce a clear result.

<u>Effects of Protein and Temperature on Spore Germination.</u> Another test was run to check the higher recovery levels obtained with a 1-percent Tween 80 solution and to confirm the possible germination-stimulating effect of the presence of protein and environmental shock on spores. The following recovery solutions were used:

1. 1% Tween 80 in distilled water
2. Distilled water
3. 1% peptone water

Distilled water was used to compare the solutions so that there would be no chance of protein matter in solutions a and b. All solutions were at room temperature when used. The experimentation followed the previous outline except that all strips were left in the recovery media for 1½ hours before ultrasonication. All strips were plated out in 20 ml TSA, incubated, and counted to determine the retention of the bacteria. These strips were not rinsed after removal from the various recovery media.

The number of bacteria grown from the heat shocked samples of room temperature peptone, which were allowed to remain for 1½ hours before ultrasonication, is 29 percent of the total number of bacteria grown from non-heat shocked samples. In a similar test with the samples grown from peptone solution kept at 4°C, the heat shocked samples showed a growth equal to 38 percent of the non-heat shocked samples.

TABLE I. NUMBER OF COLONIES PER SQUARE INCH ON STAINLESS STEEL IN 1-PERCENT TWEEN 80 RECOVERY SOLUTION (24-, 48-, and 72-hour counts)

No.	24 Hours		48 Hours		72 Hours	
	Non-Heat Shocked	Heat Shocked	Non-Heat Shocked	Heat Shocked	Non-Heat Shocked	Heat Shocked
1	124	102	133	102	133	107
2	212	77	266	78	GT*	78
3	159	92	250	92	GT	92
4	114	124	211	124	GT	124
5	87	57	172	61	GT	68
6	142	108	181	108	GT	111
7	185	108	276	110	GT	126
8	169	96	350 (2 clumps)	106	GT	123
9	174	100	221	127	221	127
10	180	121	267	162	GT	190
11	127	55	211	79	211	94
12	179	103 1 small clump clearly = 12	243	105	243	105
13	149	143	216	GT	GT	GT
14	142	90	189	96	GT	103
15	215	107	228	137	GT	137
16	125	125	173	125	GT	142
17	211	147	211	179	211	194
18	234	54	272	55	GT	55
19	237	80	255	147	GT	169
20	174	120	215	205	GT	225
Totals	33390	20090	45400	23410	45400	25130
Average	1669.5	1005	2270	1170	2270	1256.5

The average colony count from each set of plates is multiplied by a factor of 10 to compensate for the dilution factor and give an accurate number of bacteria per stainless steel strip. (NASA Standard Procedures for the Microbiological Examination of Space Hardware, 1 June 1966.)

* Grown Together

TABLE II. NUMBER OF COLONIES PER SQUARE INCH ON STAINLESS STEEL STRIPS IN 0.1-PERCENT TWEEN 80 RECOVERY SOLUTION (24-, 48-, and 72-hour counts)

No.	24 Hours		48 Hours		72 Hours	
	Non-Heat Shocked	Heat Shocked	Non-Heat Shocked	Heat Shocked	Non-Heat Shocked	Heat Shocked
1	35	110 cl*	883	144	84	GT
2	99	5	115	14	115	15
3	85-2 cl	50 cl	GT	1 clump 43	GT	67
4	191-cl	10	GT	21	GT	25
5	118	4	139	6	140	6
6	119	1	130	8	130	15
7	108-3 cl	13	108	37	GT	53
8	169-3 cl	0	206	20	GT	22
9	79	1	GT	23	GT	GT
10	90-cl	86	131	113	GT	132
11	59 cl	35	59	96	GT	115
12	109 cl	10	152	31	152	43
13	125 cl	25	225	49	GT	58
14	67 cl	13	92	35	93	42
15	157 cl	0	274	16	GT	20
16	91	10	172	24	175	39
17	76	6	96	20	GT	GT
18	95	11	106 cl	22	GT	31
19	148 cl	9	148	24	GT	32
20	114 cl	7	115	16	GT	25
Total	21360	4060	27060	7670	27120	8960
Average	106.8	203	1353	381	1356	448

* cl - clumps

TABLE III. NUMBER OF COLONIES PER SQUARE INCH ON STAINLESS STEEL STRIPS IN PEPTONE 4°C RECOVERY SOLUTION (24-, 48-, and 72-hour counts)

No.	24 Hours		48 Hours		72 Hours	
	Non-Heat Shocked	Heat Shocked	Non-Heat Shocked	Heat Shocked	Non-Heat Shocked	Heat Shocked
1	120	54	120	60	120	60
2	131	41	135	49	135	53
3	92	24	99	46	99	46
4	47	43	48	48	48	48
5	71	26	83	26	85	33
6	149	42	149	50	149	53
7	111	44	GT	45	111	45
8	158	45	GT	46	158	46
9	84	8	89	17	99	17
10	110	17	125	31	125	31
Total	10730	3440	11020	412	11290	4320
Average	1073	344	1100	412	1129	432

TABLE IV. NUMBER OF COLONIES PER SQUARE INCH ON STAINLESS STEEL STRIPS IN PEPTONE 23° C RECOVERY SOLUTION (24-, 48-, and 72-hour counts)

No.	24 Hours		48 Hours		72 Hours	
	Non-Heat Shocked	Heat Shocked	Non-Heat Shocked	Heat Shocked	Non-Heat Shocked	Heat Shocked
1	68 Growth all around edge	65	GT	65	GT	65
2	73	27	78	47	GT	47
3	132	62	GT	73	GT	73
4	176	55	176	GT	176	95 GT
5	64	47	GT	56	64	56 GT
6	83	20	GT	GT	83	20 GT
7	122	52	127	GT	127	52 GT
8	93	10	GT	11	93	13
9	65	43	91	GT	91	43 GT
10	151	47	GT	GT	151	47 GT
Total	10270	4280	10320	4690	10630	4810
Average	1027	428	1032	469	1063	481

DISCUSSION OF RESULTS

Recovery Media

The counts for heat shocked plates were consistently lower than for the non-heat shocked plates. The higher recovery levels (as with 1-percent Tween 80 solution) give a 2 to 1 recovery rate. There are many possible reasons for this. A prime consideration is that ultrasonication may affect spores in liquid media more in relation to the amount of protein in the recovery media. The greater amounts of protein in media apparently increase in spores' resistance to disintegration by ultrasonic energy [2]. Therefore, spores in recovery media lacking protein (distilled water, Tween 80 solution, weak peptone suspensions) might be more affected by this energy.

Both spores and vegetative forms of Bacillus subtilis are more resistant to heat in dry material than they are when heated in dilute aqueous solutions. The greater number of bacteria in such a solution, either spores or vegetative cells, gives greater resistance also [3].

From the above, we see that the growth of 50 percent of the spores indicates that heat shock effect on spores is insignificant.

The final test, in which distilled water was used to eliminate proteins in the solution, showed a much lower level of growth than previous tests (particularly those with Tween 80 in deionized water). Both ultrasonication and heating have a more severe effect on spores if distilled water is used [4].

Heat Shock Effect

To indicate that the heat shocked plate counts do not represent spores that require the stimulation of heat to germinate, the average number of heat shocked and non-heat shocked colonies from the 1-percent Tween 80 solution were combined. The total number of bacterial colonies (3.53×10^3) exceeded the number of bacteria initially placed on the strips (2.46×10^3). The need for heat shock as a means of inducing germination in spores does not exist.

Level of Recovery

The retention of bacteria on the strips seems to be much less for solutions in which Tween 80 is added, as shown in Table V. This nonionic detergent appears to reduce surface tension and to substantially increase removal of bacteria by ultrasonication. The low level of retention is illustrated in Figure 1 by a typical strip with a growth of only 10 colonies.

TABLE V. COMPARATIVE NUMBER OF COLONIES RECOVERED FROM SAMPLES AND PLATES STRIPS

Recovery Solution	Plates	Strip
Distilled Water	1.82×10^3 (clumped)	2.29×10^3
Peptone Water	1.38×10^3	9.9×10^2
1% Tween 80	1.73×10^3	1.2×10^2

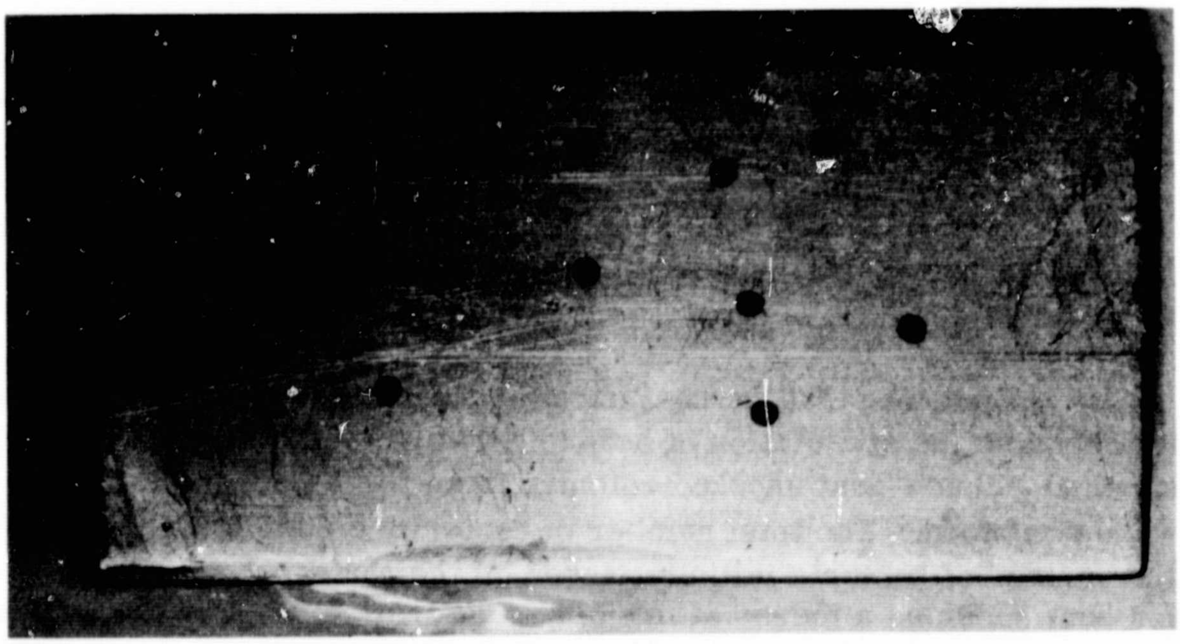

FIGURE 1. COLONY GROWTH FROM 1-PERCENT TWEEN 80 AS A RECOVERY SOLUTION

The growth pattern on all the strips indicated that the bacteria remain on the strip primarily because of surface retention of the dried contaminating solution. The pattern in which the milliliter of contaminating solution dried on the strips is identical to growth patterns. This is particularly clear in strips plated from distilled water (Fig. 2) or from peptone (Fig. 3) for which retention level is greater.

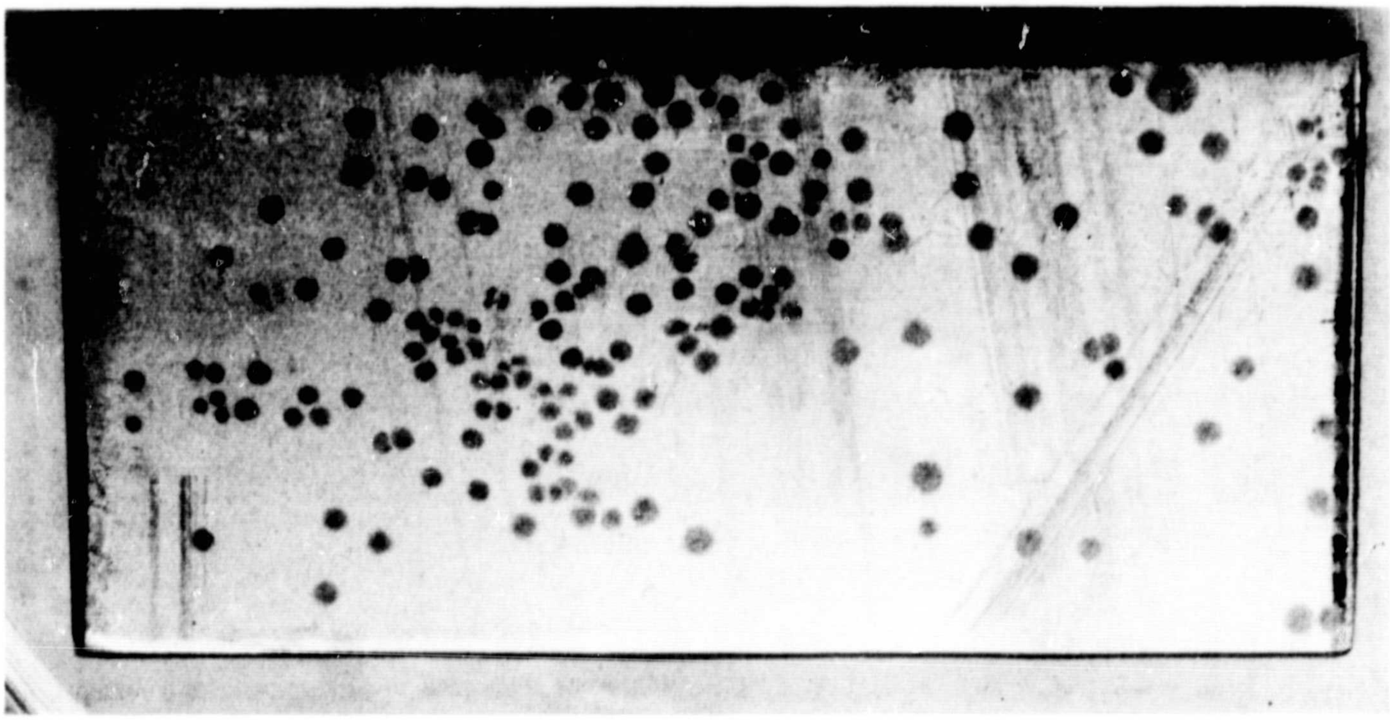

FIGURE 2. COLONY GROWTH FROM DISTILLED WATER AS A RECOVERY SOLUTION

Counting Periods

Counts were made at 24-, 48-, and 72-hours after incubation to compare the percentage of increase during the final 24 hours. This was done to determine the necessity for the 72-hour counts.

The average percentile difference between 48-hour counts and those at 72-hours in 3.08 percent for non-heat shocked samples based on individual differences shown in Table VI. The results from heat shocked samples show a 6.98 percent average difference, indicating that these samples take longer for complete germination.

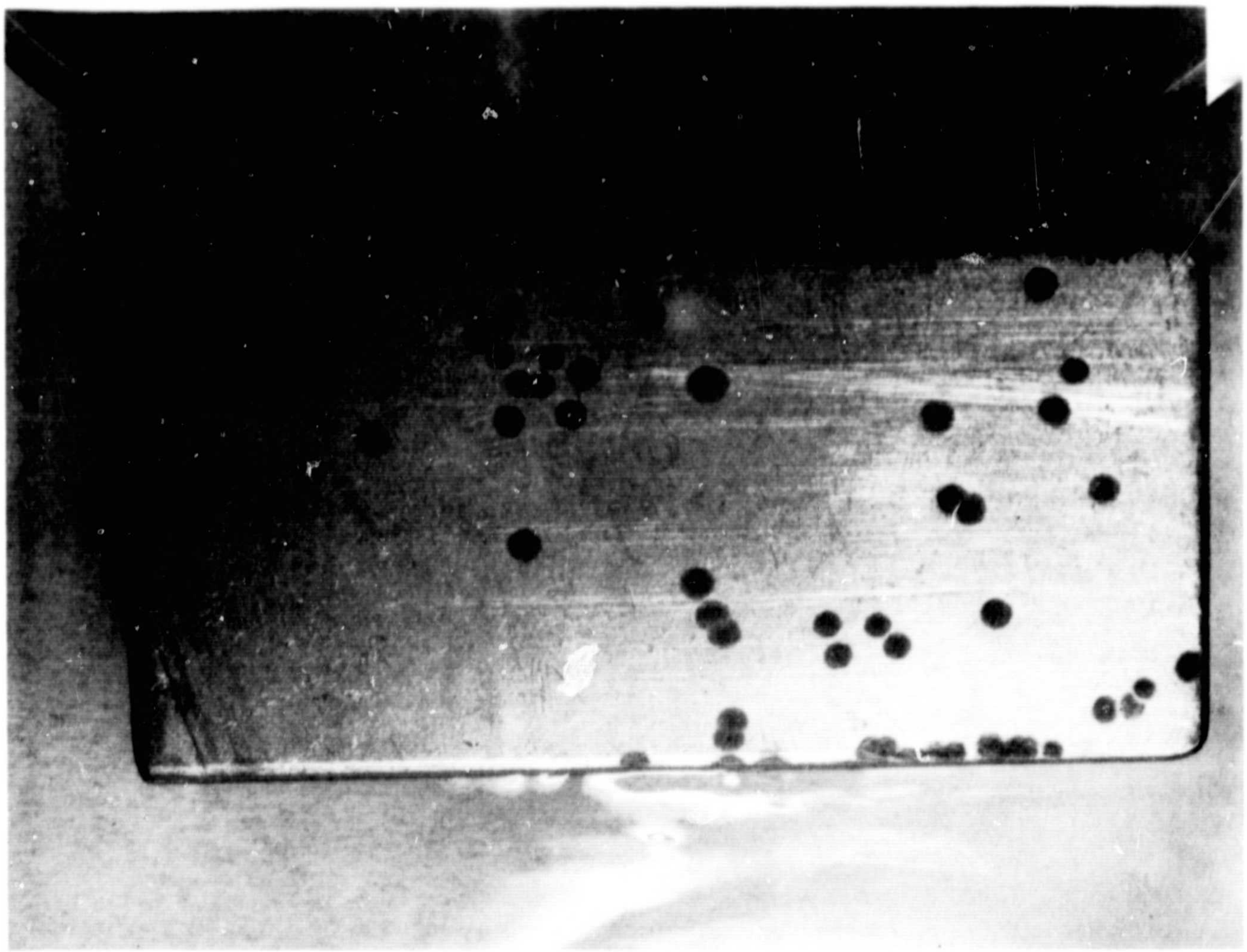

FIGURE 3. COLONY GROWTH FROM PEPTONE WATER AS A RECOVERY SOLUTION

TABLE VI. PERCENTAGE OF CHANGE BETWEEN 48- AND 72- HOUR COUNTS FOR NON-HEAT SHOCKED AND HEAT SHOCKED SAMPLES

Recovery Solution	Non-Heat Shocked	Heat Shocked
1% Tween 80	0%	7.1%
Peptone (37°C)	2.9%	2.0%
Peptone (4°C)	2.4%	4.7%
0.1% Tween 80	0.2%	15.5%
Distilled Water	4.8%	10.9%
1% Tween 80 in Distilled Water	10.6%	6.3%

CONCLUSIONS

The results from the experiments performed in carrying out this project indicate that this basic method of controlled contamination is more effective than the adaption of the stainless steel strip analysis.

The use of a solution of 1-percent Tween 80 deionized water provided a consistently higher level of recovery, as well as elimination of the problem of germination and growth in recovery media. Although heat shocked samples are reduced in number, there are many factors which may affect these samples. A 50-percent survival rate for spores is a significant level of viable recovery. Germination will occur without the stimulus of heat shock, as shown by the high level of recovery for samples that are not heat shocked. Therefore the heat shock step is not required.

To reiterate, this method of controlled contamination should be used to determine the specific effectiveness of sterilization or decontamination procedures in reducing bioloads.

PROSPECTIVE PROCEDURES

Procedure for Controlled Level of Contamination to Determine Changes in Bioload by Sterilization or Decontamination

1. Parts to be sterilized should be wrapped in Kraft paper or aluminum foil and labeled. These parts should be subjected to a dry heat sterilization cycle, $24\frac{1}{2}$ hours at 135° C.

2. Using a class 100 working area, clean bench, or clean room, place each part in a sterile, labeled petri dish. Ultrasonicate the contaminating solution for 5 minutes to eliminate clumping of spores. Using sterile 1 ml pipettes, place 1 ml of the suspension on each part, having petri dishes open to air-dry in the work area.

3. After the solution has dried on the parts (approximately 10 minutes), place each part in a labeled screwcap jar containing 60 ml of 1-percent Tween 80 solution suspended in deionized water. For larger parts, a greater amount of recovery solution would be used. These jars are ultrasonicated for 12 minutes according to NASA Standard Procedures for Microbiological Examination of Space Hardware, 1 June 1966. Two modifications of this technique could be used to provide more accurate results: (1) using a framework to keep bottle tops our of the bath solution, and (2) maintaining the bath solution composition at 0.3-percent Tween 80 in deionized water.

4. Pipette two 6-ml samples from each jar into sterile petri dishes. Add 20 ml molten TSA. Allow to solidify; invert, and incubate at 37°C for 48 hours.

5. Count at 24- and 48-hours (Fig. 4).

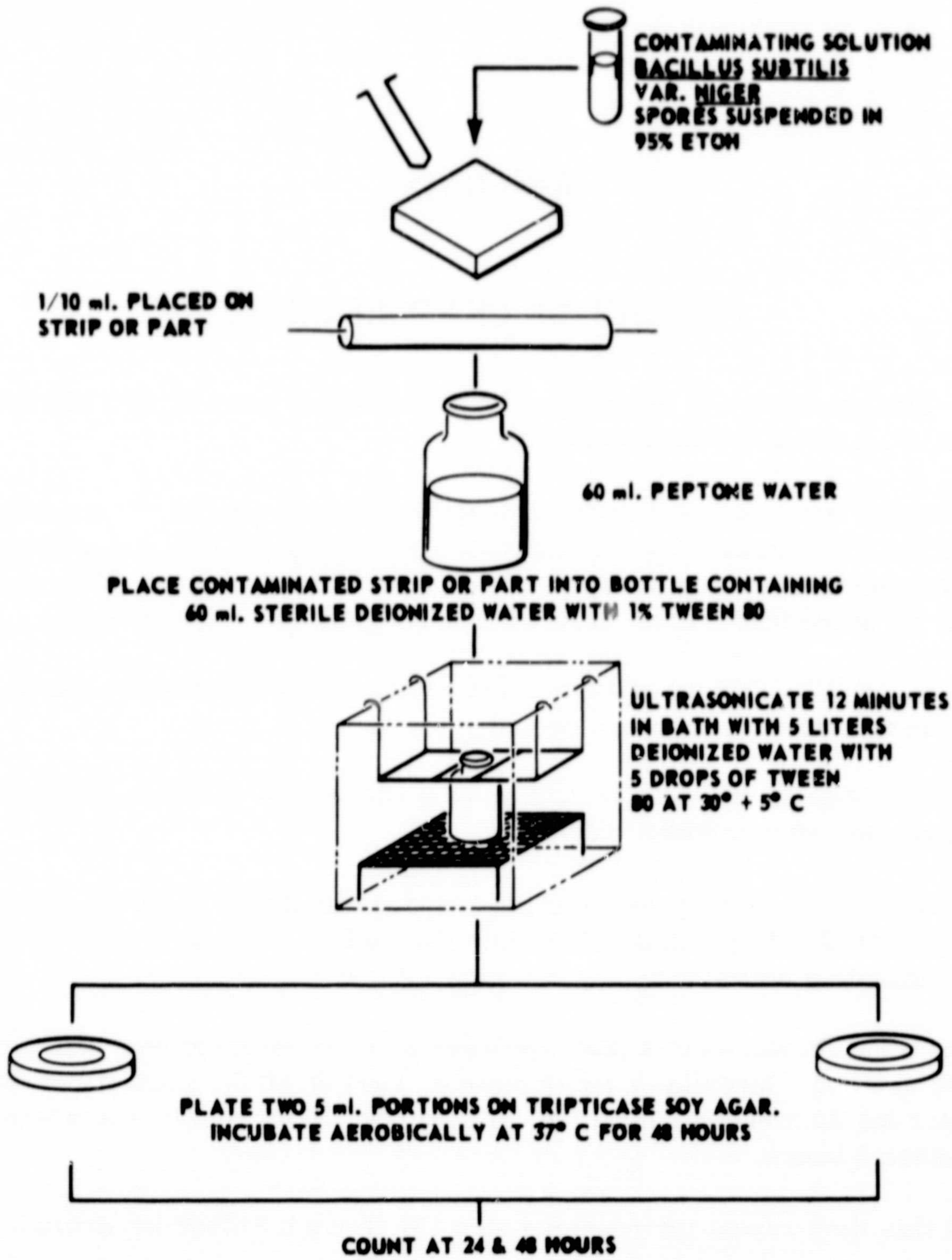

FIGURE 4. SCHEMATIC OUTLINE OF PIECE PART AND STAINLESS STEEL STRIP ANALYSIS USING ALCOHOL SPORULATION METHOD

APPENDIX

PRODUCTION OF SPORE CROPS

This procedure is an adaption of the technique used by Dr. Martin S. Favero, U. S. Public Health Service.

1. Using an aqueous culture with at least 50-percent spores, heat shock at 80° C for 15 minutes. Swab to inoculate the surface of 100- by 15-mm plate of Thermoacidurans Agar Modified (TAM) sporulation medium. This medium is made up according to directions with these additions before pouring:

a. $CaCl_2$: 0.8 ml of 10-percent solution, filtered and sterilized, per liter; add after medium has cooled.

b. Magnesium sulfate: 0.2 ml of a 10-percent solution, filtered and sterilized; add after medium has cooled.

2. Prepare a heavy suspension of these spores in sterile distilled water; heat shock at 80° C for 15 minutes. Inoculate the surface of another plate of solidified TAM; incubate 3 hours.

3. Wash the growth on this plate; prepare a suspension and heat shock for 15 minutes at 80° C. Inoculate a fresh plate of TAM at 41° C (place fresh plate in incubator for 30 minutes before inoculation) and incubate at this temperature for another 3 hours.

4. At this time repeat the previous step (3) through 3-hour incubation.

5. This culture is now in the log phase of growth. Use to inoculate 10 plates of TAM. Incubate at least 24 hours. Check each plate for sporulation using phase scope or spore stain. The Wirtz-Conklin Method (Melltver and Bartholemew's "Cold" Method) is quite effective.

a. Fix the smear by passing through the flame 20 times.

b. Stain 10 minutes with saturated aqueous malachite green (7.6-percent) without heat.

c. Rinse with tap water about 10 seconds.

d. Stain 15 seconds in 0.25-percent aqueous safranin.

e. Rinse, blot, and dry.

Results: Spores, green; rest of cell, red.

Discard any culture which does not have at least 90-percent sporulation after 48 hours.

6. Collect these spores from the plates using cold (4°C), sterile, distilled water. Ultrasonicate the suspension in the presence of sterile glass beads for 30 minutes.

7. Centrifuge the suspension (10 000 RPM) so that spores will separate from cellular debris and form a light layer at the surface. Wash the suspension seven times in cold, sterile, distilled water. Wash twice in ethanol before storage in 95-percent ethanol at 4°C in screwcap test tubes.

This spore crop may be stored at 4°C for at least 30 days without loss of viability. During this time a series dilution may be prepared to check the number of spores. This is done in accordance with Volume III: Procedures Manual for Planetary Spacecraft to be Sterilized by Heating. Dilute spore suspension to level of 10^2 spores per milliliter. Retain for use as contaminating solution.

REFERENCES

1. Jacobs, Morris B.; and Gerstein, M. J.: Handbook of Microbiology. D. Van Nostrand Co., Inc., Princeton, N. J., 1960.

2. Culture Collections: Perspectives and Problems. Proceedings of the Specialists Conference on Culture Collections, Ottawa, August 1962. Edited by S. M. Martin, University of Toronto Press, 1963.

3. Knoupsi, Georges: The Endospore of Bacteria. Microbiological Review, vol. 12, March 1948, pp. 19-77.

4. Kruger, Walter W.; and Johansson, Karl R.: Principles of Microbiology. W. B. Saunders Co., Philadelphia, 1959.

BIBLIOGRAPHY

Collins, C. H.: Microbiological Methods. Butterworth's, London, 1964.

General Electric Spacecraft Department, Procedures Manual for Planetary Spacecraft to be Sterilized by Heating. Vol. III, Biological Handbook for Engineers. Preliminary Publication, 31 March 1967.

Gould, G. W.; Hutchens, A. D.; and King, W. L.: Function and Location of a Germination Enzyme in Spores of B. Cereus. Journal of General Microbiology, vol. 44, 1966, pp. 293-302.

Maynell, G. G.; and Meiynell, Elinor: Theory and Practice in Experimental Bacteriology. Cambridge University Press, England, 1965.

Munell, W. G.; and Scott, W. J.: The Heat Resistance of Bacterial Spores at Various Water Activities. Journal of General Microbiology, vol. 43, 1966, pp. 411-425.

NASA Standard Procedure for the Microbiological Examination of Space Hardware, 1 June 1966.

Oginsky, Evelyn L.; and Umbert, W. W.: An Introduction to Bacterial Physiology. W. H. Freinman & Co., San Francisco, 1959.

Recent Progress in Microbiology. Symposium at VIII International Congress for Microbiology, 1962, University of Toronto Press, 1963.

Rose, Anthony H.: Industrial Microbiology. Butterworth's, London, 1961.

Salle, A. J.: Fundamental Principles of Bacteriology. McGraw-Hill Book Co., Inc., N. Y., 1961.

Stanier, Roger Y.; Dandaroff, Michael; and Edelburg, Edward A.: The Microbial World. Prentice-Hall, Inc., Englewood Cliffs, N. J., 1963.

BIBLIOGRAPHY (Concluded)

Weiser, Harry H.: Practical Food Microbiology and Technology. The AVI Publishing Co., Inc., Westport, Conn., 1962.

Wolfe, J.; and Thorley, C. M.: The Effects of Various Germination Agents on the Spores of Some Strains of B. subtilis. Journal of Applied Bacteriology, vol. 20, 1957, pp. 384-388.